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Recovery of the proteose peptone component 3 from cheese whey in Reppal PES 100/polyethylene glycol aqueous two-phase systems

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Abstract

Recovery of the proteose peptone component 3 from cheese whey was optimal using a 16% (w/w) Reppal PES 100 – 24% (w/w) PEG 600 aqueous two-phase system, at pH 7, giving a mass recovery yield of 99% and a purity of 83% for proteose peptone component 3 in the upper phase. Using the above system a partition coefficient of 30.7 and a purification factor of 6.9 were achieved.

Abbreviations: HPL – hydrophilic proteose peptone, PP3 – proteose peptone component 3, TPP – total proteose peptone, WPC – whey protein concentrate.

Introduction

The potential exploitation of selected milk proteins as ingredients in functional food products has been the reason for an increasing interest in their fractionation (Andrews *et al*. 2000). Heating of skimmed milk (95 °C, 30 min) followed by acidification promotes the denaturation of whey proteins and their co-precipitation with caseins, which are insoluble at pH 4.6 (Girardet & Linden 1996). In spite of these drastic conditions, a heterogeneous fraction called proteose peptone, remains soluble. The total proteose peptone (TPP) fraction of bovine milk represents about 10% of total whey protein. Its principal components have been designated as components 3, 5 and 8 (PP3, PP5, PP8) (Innocente *et al*. 1999). The PP3 is extremely hydrophobic and particularly interesting because of its functional properties, such as its emulsifying power, strong affinity for oil-water interface, strong foaming properties and biochemical role (Innocente *et al.* 1999).

Aqueous two-phase systems (ATPS) have found widespread use for separation and purification of biological products (Cascone *et al.* 1991). The most widely used system consists of dextran and polyethylene glycol (PEG), but the high cost of fractionated dextran has limited its use for large-scale applications. However, some other polysaccharides, such as hydroxypropyl starch, appear successful replacements providing at least a 42% reduction in cost (Venâncio *et al.* 1996). ATPS have previously been applied to separate the major proteins from cheese whey (Rodrigues *et al.* 2001, Alves *et al.* 2000). Cheese whey has a high protein content (12% w/w; dry basis), with a minor protein fraction (1.2% w/w; dry basis) consisting of proteose peptone (Alves *et al.* 2000).

In this work, systems composed of PEG and Reppal PES 100 (a commercial hydroxypropyl starch) were studied to assess the recovery of the PP3 from whey protein concentrate (WPC). The main factors affecting the PP3 partition behaviour were evaluated using a purified PP3 fraction. The results were then used to assay the PP3 partition from the TPP fraction of a 80% (w/w) WPC.

Methods

Materials

PEG with average molecular masses of 600, 900 and 8000 Da were purchased from Sigma; PEG with average molecular masses of 1500, 4000, 6000 and 10 000 Da was purchased from Merck. Reppal PES 100 was obtained from Reppe AB, Växjö, Switzerland. WPC with an 80% (w/w; dry basis) protein content was obtained from Quinta dos Ingleses, Lda.

Extraction of total proteose peptone (TPP) fraction

The TPP fraction was prepared from WPC by heating (95 °C, 30 min) and precipitation at pH 4.6 (by 1 M HCl). Precipitation from the filtrate involved using 50% saturated $(NH_4)_2SO_4$ and the TPP was separated using a centrifuge (5000 × *g* for 30 min). After it was exhaustively dialysed and then lyophilised (Innocente *et al.* 1999). A yield of approx. 2 g of TPP fraction was obtained from 200 g of WPC.

Purification of the PP3 by FPLC

Proteose peptone fractionation was performed on an Octyl Sepharose 4FF column (Supelco) connected to an FPLC system (Pharmacia). A freeze-dried TPP fraction (150–200 mg) diluted in 1 M phosphate buffer (pH 7) was loaded onto the column equilibrated in the same buffer. The flow rate was 1.5 ml/min and detection was at 280 nm. The first fraction containing hydrophilic components was eluted in saline buffer, whilst the hydrophobic fraction (PP3) was eluted at the end in pure water. After dialysis, the purified PP3 was freeze-dried (Innocente *et al.* 1999, 2002).

Two-phase systems

Reppal PES 100 and PEG (molecular masses: 600, 900, 1500, 4000, 6000, 8000 and 10 000) stock solutions were prepared at 50% (w/w) in distilled water. For all assays the Reppal PES 100 composition of the systems was 12 and 16% (w/w). For preparation of systems with purified PP3 or TPP fractions, polymer stock solutions were weighed and mixed with distilled water, phosphate buffer (final concentration 10 mM, pH values of 3, 5, 7 or 9) and protein solution (purified PP3 or TPP fraction). The total mass of the systems was adjusted to 1 g or 10 g with distilled water for the purified PP3 and TPP fraction respectively. Phases were separated by centrifuging (5 min, 4000 × *g*) with the total mass and protein content determined in both phases.

Table 1. PEG molecular mass effect on PP3 partition. The partition parameters were determined as the average of 3 independent assays. The error determined for both partition parameters was 5%.

PEG molecular mass	KPP3[a]
10 000	1.4
8000[1]	1.8
6000[1]	2.3
4000[1]	4.3
1500[2]	4.3
900[2]	9.3
600[2]	20.9

[1]PEG concentration – 12% (w/w)
[2]PEG concentration – 22% (w/w)

[a]KPP3 – PP3 partition coefficient, defined as the ratio between the PP3 upper and lower phase concentrations.

Protein assays

The protein concentration of the purified PP3 in both phases was determined using the Bradford method.

In the assays performed using the TPP fraction, the PP3 partition behaviour was evaluated using hydrophobic chromatography. The samples (500 μl) were injected into an Octyl Sepharose 4 FF column (Supelco). Assays were run at a flow rate of 1.5 ml min^{-1}, room temperature, using 1 M phosphate buffer at pH 7, with a gradient (1–0 M) as eluent. The absorbancy at 280 nm was recorded using a L-7455 Diode-Array detector (Merck), and analysed with D-7000 HPLC System Manager (Version 3.1) Software.

Results and discussion

PP3 partition in Reppal PES 100-PEG systems

PEG molecular mass effect on PP3 partition

Table 1 shows the effect of the molecular mass of PEG on the purified PP3 partition. As all the values obtained are higher than 1, this indicates that, in the range of molecular masses tested, PP3 is mainly found in the upper phase of the system. This is not unexpected, considering the hydrophobic properties of both

Table 2. Reppal PES 100 concentration and pH effects on PP3 partition. The partition parameters were determined as the average of 3 independent assays. The error determined for both partition parameters was 5%.

12% w/w Reppal PES 100 12% w/w PEG 4000 (pH)	KPP3[a]
3	0.8
5	3.9
7	4.3
9	14.8
Reppal PES 100 12% w/w PEG 4000, pH 7 (concentration)	KPP3[a]
12% w/w	4.3
14% w/w	6.6
16% w/w	12

[a]KPP3 – PP3 partition coefficient, defined as the ratio between the PP3 upper and lower phase concentrations.

PEG and PP3 (Franco *et al.* 1996). It is also clear that the partition coefficient of the PP3 decreased with increasing PEG molecular mass. This is most probably due to an exclusion effect that for higher PEG molecular mass causes PP3 to partition more in the lower phase. In order to test a range of PEG molecular masses two values of PEG concentration were chosen [12 and 22% (w/w)] because, as it was observed for PEG 4000, at the 22% (w/w) PEG concentration, PP3 precipitation occurred.

pH effect on PP3 partition

The evaluation of the effect of pH upon the PP3 partition is given in Table 2. The effect of pH on separation was such that an increase in pH leads to an increase in PP3 partition to the upper phase. As the pH influences the overall protein charge it is therefore expected that at values of pH lower than the isoelectric point (in this case in the range 4.9–6.1), the protein has an overall positive charge and will move into the lower phase. Above the isoelectric point the protein should be accepted more favourably by the PEG phase. At pH 9, the high value observed for protein partition can be attributed to protein precipitation (visually observed).

Table 3. PEG concentration effect on PP3 partition. *Composition of the system*: 16% (w/w) Reppal PES 100 and PEG, at pH 7. The partition parameters were determined as the average of 3 independent assays. The error determined for both partition parameters was 5%.

System	KPP3[a]	YPP3,U(%)[b]
PEG 600		
20% w/w	19.3	98.7
22% w/w	20.9	98.9
24% w/w	28.7	99.2
PEG 900		
20% w/w	6	96
22% w/w	9.3	97.4
24% w/w	14.8	98.3
PEG 1500		
20% w/w	3.8	93.8
22% w/w	4.3	94.5
24% w/w	3.8	93.6

[a]KPP3 – PP3 partition coefficient, defined as the ratio between the PP3 upper and lower phase concentrations.
[b]YPP3,U – mass recovery yield of PP3 in the upper phase, defined as $YPP3, U = \frac{100}{1+\frac{1}{R}\times\frac{1}{KPP3}}$, being R the ratio between upper and lower phase volumes.

Reppal PES 100 concentration effect on PP3 partition

The effect of Reppal PES 100 concentration was evaluated and from the results given in Table 2 is possible to observe that the highest Reppal PES 100 concentration gave the highest PP3 partition coefficient, probably because of excluded volume effect. Higher concentrations of Reppal PES 100 were also tested (data not shown), but these were found to induce higher levels of protein precipitation (visual observation).

Results gathered from the PEG molecular mass, pH and Reppal PES 100 concentration effects on the PP3 partition provided preliminary data in order to establish the conditions for further optimisation of the PP3 recovery. Therefore the Reppal PES 100 was maintained at 16% (w/w), with a constant system pH of 7. The systems were made with PEG molecular masses of 1500, 900 and 600, with the PEG in the range of 20 to 24% (w/w).

PEG concentration effect on PP3 partition

The ATPS can be interpreted as being formed by two immiscible solvents with different hydrophobicities,

Table 4. PP3 partition in the total proteose peptone (TPP) fraction. All partition parameters were determined as the average of 3 independent assays. The errors determined were 8% for KPP3, 11% for KHPL and 5% for other parameters.

System: 16% (w/w)	PEG600 concentration (w/w)			PEG900 concentration (w/w)		
Reppal PES100, pH 7	20%	22%	24%	20%	22%	24%
KPP3[a]	18	27.7	30.7	10.6	12	13.6
KHPL[a]	0.6	0.4	0.2	0.1	0.1	0.1
YPP3,U (%)[b]	98.2	98.8	98.9	97	97.3	97.6
YHPL,L (%)[c]	37.8	49	64.8	70.2	70.7	71.1
PPP3,U (%)[d]	38.5	58.5	83	67	76.9	74.4
PHPL,L (%)[d]	99.9	100	100	100	100	100
PFPP3,U[e]	2.1	3.1	6.9	4.8	9.4	6.3
PFHPL,L[e]	15.6	25.7	24.5	15.5	9.3	15.8

[a]KPP3 (or KHPL) – PP3 [or hydrophilic fraction (HPL)] partition coefficient.
[b]YPP3,U – mass recovery yield of PP3 in the upper phase, defined as YPP3,U = 100/(1+1/R × 1/KPP3), being R the ratio between upper and lower phase volumes.
[c]YHPL,L – mass recovery yield of the hydrophilic fraction in the lower phase, defined as YHPL,L = 100/(1+R × KHPL).
[d]PPP3,U (or PHPL,L) – purity of PP3 (or HPL) in the upper (or lower) phase, defined as the ratio between the PP3 (or HPL) and the total protein mass yield recoveries in the upper (or lower) phase.
[e]PFPP3,U (or PFHPL,L) – purification factor of PP3 (or HPL) in the upper (or lower) phase, defined as the ratio between the protein of interest and the contaminant concentration in the final product.

which determine the protein partition. The hydrophobicity in each phase depends on the polymer concentration. Table 3 shows that as the PEG concentration increases, the PP3 partition coefficient also increases, which may result from an increase of the hydrophobic interactions in the PEG-rich upper phase (more PEG produces stronger hydrophobic interactions forcing the hydrophobic PP3 to partition to the upper phase).

Results from the assays show that a system composed of 24% (w/w) PEG 600 and 16% (w/w) Reppal PES 100 at pH 7 produced the highest partition of the PP3, giving a mass recovery yield of 99% in the upper phase and partition coefficient of 28.7.

Separation of PP3 in total proteose peptone (TPP) fraction

These assays were repeated for the TPP fraction, in order to determine the best operational conditions for the separation of PP3 from the TPP fraction, in a one-step liquid-liquid extraction. Table 4 shows the effect of PEG molecular mass and concentration on the PP3 partition. As in the above results the increase PEG molecular mass moves the PP3 into the Reppal PES 100-rich lower phase and the PEG concentration increase moves the protein into the PEG-rich upper phase.

The best separation conditions for the TPP fraction were 16% (w/w) Reppal PES 100 – 24% (w/w) PEG 600 at pH 7, confirming the values obtained in the assays performed with the purified PP3, allowing purification of PP3 in the upper phase to be achieved. This is because the PP3 exclusively partitions to the upper phase, with a small amount of hydrophilic fraction detected. These systems also allow the isolation of the hydrophilic fraction in the lower phase, which is practically free of PP3, as seen in Table 4. This table shows that the purification factors for PP3 are lower than those obtained for the hydrophilic fraction, which may result from a small amount of hydrophilic fraction that moves into the upper phase. A higher PEG concentration gives higher purification factors and could improve the separation. Nevertheless, as discussed previously an increased PEG concentration also increases protein precipitation, thus limiting PEG concentrations to below 24% (w/w).

The system composed of 24% (w/w) PEG 600 and 16% (w/w) Reppal PES 100 at pH 7, gave the highest partition of the PP3 with a mass recovery yield of 99% for PP3 in the upper phase and partition coefficient of KPP3 = 30.7. A purity of 83% for PP3 and a purifi-

cation factor (PFPP3,U) of 6.9 were obtained for this phase.

Further recovery of the PP3 from the PEG-rich phase is possible using a PEG – phosphate system. In preliminary studies, systems composed of PEG and potassium phosphate were studied for the separation and purification of the PP3 and recovery in the phosphate-rich phase could be achieved using a 25% (w/w) PEG 20 000 – 10% (w/w) phosphate system, at pH 7. A mass recovery yield of 90% for PP3 in the lower phase was obtained along with a partition coefficient of 0.5.

Conclusion

A Reppal PES 100 – PEG aqueous two-phase system proved to be an efficient and inexpensive system for PP3 recovery from cheese whey. We demonstrate that it is possible to recover the PP3 in the upper phase and to isolate the hydrophilic proteose peptone in the lower phase of these systems.

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